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## **Changes in body posture alter plasma nitrite but not nitrate concentration in humans**

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## Abstract

**PURPOSE:** This study evaluated the change ( $\Delta$ ) in plasma volume (PV), nitrate [ $\text{NO}_3^-$ ], and nitrite [ $\text{NO}_2^-$ ] concentration following changes in posture in the presence and absence of elevated plasma [ $\text{NO}_3^-$ ] and [ $\text{NO}_2^-$ ]. **METHODS:** Fourteen healthy participants completed two trials that were preceded by either supplementation with  $\text{NO}_3^-$ -rich beetroot juice (BR; total of  $\sim 31$  mmol  $\text{NO}_3^-$ ) or no supplementation (CON). Both trials comprised 30 min of lying supine followed by 2 min of standing, 2 min of sitting and 5 min of sub-maximal cycling. Measurements of plasma [ $\text{NO}_3^-$ ] and [ $\text{NO}_2^-$ ] were made by gas-phase chemiluminescence and  $\Delta\text{PV}$  was estimated using the Dill and Costill method. **RESULTS:** Plasma [ $\text{NO}_2^-$ ] decreased from baseline (CON:  $120 \pm 49$  nM, BR:  $357 \pm 129$  nM) after lying supine for 30 min (CON  $77 \pm 30$  nM; BR  $231 \pm 92$  nM, both  $P < 0.01$ ) before increasing during standing (CON  $109 \pm 42$  nM; BR  $297 \pm 105$  nM, both  $P < 0.01$ ) and sitting (CON  $131 \pm 43$  nM; BR  $385 \pm 125$  nM, both  $P < 0.01$ ). Plasma [ $\text{NO}_2^-$ ] remained elevated following exercise only in CON ( $125 \pm 61$  nM  $P = 0.02$ ). Plasma [ $\text{NO}_3^-$ ] was not different between measurement points in either condition ( $P > 0.05$ ). PV increased from baseline during the supine phase before decreasing upon standing, sitting, and exercise in both trials (all  $P < 0.05$ ). **CONCLUSIONS:** Changing body posture causes rapid and consistent alterations in plasma [ $\text{NO}_2^-$ ]. Researchers should therefore carefully consider the effect of posture when measuring this variable.

**Key Words:** beetroot juice, exercise, plasma volume

## 1. Introduction

Nitric oxide (NO) is a ubiquitous signalling molecule which is synthesised endogenously from L-arginine by NO synthases (NOS) in the presence of oxygen ( $O_2$ ) and regulates a multitude of biological processes (Forstermann and Sessa 2012). For example, NO is transiently released from endothelial cells, diffusing from the production site to smooth muscle cells, resulting in dilation of the vasculature (Ignarro et al. 1987). Nevertheless, the majority of NO does not reach its target cells due to rapid oxidation to nitrite ( $NO_2^-$ ) and nitrate ( $NO_3^-$ ) (Kelm 1999).

However, when  $pO_2$  is reduced endogenous NO synthesis is diminished and can result in a reduced blood flow to the periphery (Lundberg et al. 2008). Under these conditions it has been shown that NO metabolites can be utilised as a reservoir for NO production via the  $NO_3^-$  -  $NO_2^-$  - NO pathway. Firstly,  $NO_3^-$  can be reduced to  $NO_2^-$  by facultative bacteria on the tongue (Duncan et al. 1995) via the entero-salivary system (Lundberg and Govoni 2004). The resultant  $NO_2^-$  is ingested and absorbed into the blood plasma where it may be reduced to NO under certain local physiological conditions (Lundberg et al. 1994; Millar et al. 1998; Modin et al. 2001; Castello et al. 2006). However, due to the short half-life of NO, plasma [ $NO_2^-$ ] is still considered to provide the best approximation of vascular NO bioavailability (Kelm 1999; Lauer et al. 2001). Increased plasma [ $NO_2^-$ ] is also associated with improved endothelial function (Rassaf et al. 2006) and superior exercise capacity (Totzeck et al. 2012) and is therefore routinely measured in cardiovascular and exercise science research.

$NO_3^-$  is also readily available within our diet and its consumption has been shown to significantly increase plasma [ $NO_3^-$ ], and thus [ $NO_2^-$ ]. There is now an abundance of research which has explored the potential therapeutic and ergogenic benefits of supplementation with dietary  $NO_3^-$  (Siervo and Lara 2013; Pawlak-Chaouch et al. 2016; McMahon et al. 2017). This

research typically encapsulates repeated measurements of physiological variables during periods of rest and exercise. During the experimental protocols, blood samples may be routinely collected while participants are either supine (Muggeridge et al. 2015), seated (Sandbakk et al. 2015), or standing (Wylie et al. 2013b). However, it is well-established that alterations in posture lead to marked changes in plasma volume (PV) that can alter the molecular concentrations of common biochemical analytes (Thompson et al. 1928; Fawcett and Wynn 1960; Hagan et al. 1978; Hagan et al. 1980; Lippi et al. 2015). A probable explanation is that postural-induced alterations in hydrostatic pressure force fluid and protein to shift between the blood and interstitial space (Cohn 1966; Stokke et al. 1986). Lippi and colleagues (2015) report that PV was decreased by 3% and 14% when moving from a supine posture to sitting and standing, respectively. Furthermore, short duration high-intensity exercise has been shown to decrease PV between 5-22 % in various exercise modalities (Kargotich et al. 1998).

Previous research has shown considerable variability in the values of basal and supplemented plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  (Wylie et al. 2013b; Muggeridge et al. 2015; Sandbakk et al. 2015; McMahon et al. 2017). Given that  $\text{NO}_3^-$  is measured in the micromolar range and  $\text{NO}_2^-$  in the nanomolar range, the measured concentration of these variables may be subject to postural-induced changes. Indeed, we have recently shown that plasma  $[\text{NO}_2^-]$  declined significantly over a 30 min period while participants lay supine (Muggeridge et al. 2015), suggesting that posture and PV shifts may alter plasma  $[\text{NO}_2^-]$ . However, several factors exist which may account for these observations. For example, prolonged sitting is known to decrease shear stress, whereas standing may increase shear stress and endogenous NO production (Uematsu et al. 1995; Sessa 2004; Hsieh et al. 2014; Restaino et al. 2016; Morishima et al. 2017). This

demonstrates that further research is required to determine the impact of posture on NO metabolites.

No study has explored the effects of postural changes on the measured concentrations of plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$ . The aim of the present study, therefore, was to determine the magnitude of the postural-induced changes in plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  and PV at rest and following short-duration exercise. The experiment was conducted both with and without prior dietary  $\text{NO}_3^-$  supplementation. We hypothesised that postural changes would alter plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  which would be inversely associated with the change in PV.

## **2. Methods**

### **2.1. Participants**

Fourteen healthy and recreationally active participants (9 males and 5 females, age  $27 \pm 4$  years, stature  $176 \pm 7$  cm, and body mass  $71 \pm 11$  kg) volunteered to participate in the study. Written informed consent was obtained from all individual participants included in the study. The study was approved by the School of Science and Sport Ethics Committee at The University of the West of Scotland and all procedures were performed in accordance with the 1964 Declaration of Helsinki and its later amendments.

### **2.2. Study design**

Each participant attended the laboratory on two separate occasions with a minimum of six days between each visit. The experimental conditions were identical in each visit with the exception that the first trial was conducted with no dietary intervention (control; CON). The second was

preceded by ingestion of 3 x 70 ml of NO<sub>3</sub><sup>-</sup>-rich beetroot juice (Beet it, James White Drinks, UK) the day before and 2 x 70 ml, 2 h before the first blood sample (BR; total of ~31 mmol NO<sub>3</sub><sup>-</sup>). Participants recorded their diet 24 h prior to CON and were asked to repeat this as closely as possible prior to BR. All trials were completed before 11 a.m. at the same time of day for each participant and following an overnight fast. Participants were instructed to avoid caffeine, foods high in NO<sub>2</sub><sup>-</sup> and NO<sub>3</sub><sup>-</sup> (e.g. green leafy vegetables and cured meats), alcohol, mouthwash, and strenuous exercise 24 h prior to the experiment. Participants were provided with one bottle of drinking water (Harrogate, UK) prior to CON trial and given instructions to arrive at the lab well hydrated. Participants recorded the volume of water ingested prior to CON and matched the volume prior to BR.

### 2.3. Procedures

Following standard anthropometric measurements (stature and body mass), participants lay in a supine posture to allow for the insertion of a cannula into the antecubital vein. Following cannulation, participants stood up for 10 min prior to lying supine to start the experimental trial. Baseline measurements (0 min) of venous blood, blood pressure (BP) and heart rate (HR) were recorded immediately. The measurement of BP was conducted using an automated sphygmomanometer (Omron M10, Kyoto, Japan) in triplicate during supine measures and in duplicate for the standing and seated measures. Mean arterial pressure (MAP) was calculated by the following equation:

$$\text{MAP} = (2 \times \text{diastolic BP} + \text{systolic BP}) / 3$$

Continuous measurement of HR was conducted using telemetry (Polar electro, Oy, Finland). Participants lay supine for a total of 30 min followed immediately by 2 min of standing, 2 min of sitting, and then 5 min of cycling at 60% of the age-predicted maximal heart rate. The duration of the standing, sitting, and exercise phases was kept brief to minimise pharmacokinetic alterations in plasma  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  in the BR trial. Plasma  $[\text{NO}_2^-]$  and  $[\text{NO}_3^-]$  are known to peak at ~2.5 h and ~1.5 h, respectively (Webb et al. 2008; Lundberg and Weitzberg 2009; Larsen et al. 2010; Wylie et al. 2013a; McIlvenna et al. 2017). Therefore, the end of the supine phase was designed to coincide with the peak in plasma  $[\text{NO}_2^-]$ . The experimental protocol reflects many exercise physiology research studies that incorporate both resting and exercise phases (Wylie et al. 2013b; Muggeridge et al. 2015; Sandbakk et al. 2015).

Collection of venous blood, BP and HR were repeated throughout the experiment as detailed in Figure 1. The measurement of BP was not made during exercise due to difficulties in obtaining a stable measurement. Venous blood was collected in 10 ml aliquots and the cannula flushed with sterile 0.9% saline solution between samples to keep the line patent. Whole blood was initially separated into EDTA vacutainers (BD Vacutainer). One vacutainer was refrigerated at 4°C for the later analysis of haemoglobin concentration and haematocrit. All samples were analysed within 6 h. The other vacutainer was centrifuged at 4000 rpm and 4°C for 10 min within 3 min of collection (Pelletier et al. 2006; Bailey et al. 2009). The plasma was then separated, frozen at -80 °C, and analysed within 4 months of initial collection for determination of  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$ .



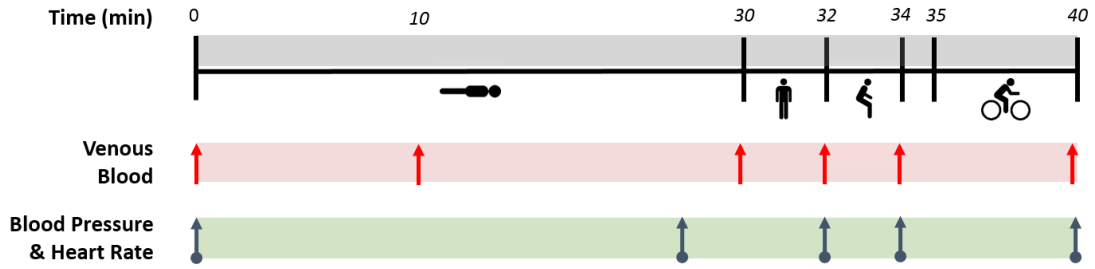


Fig. 1. Schematic of measurement time points for CON and BR trials.

#### 2.4. Plasma Nitrite and Nitrate analysis

Measurements of  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  were made using ozone-based chemiluminescence (Rogers et al. 2005). For the measurement of  $[\text{NO}_2^-]$ , tri-iodide reagent (2.5 ml glacial acetic acid, 0.5 ml of 18  $\Omega$  deionised water and 25 mg sodium iodide) and 100  $\mu\text{L}$  of anti-foaming agent were placed into a customised glass purge vessel infused with nitrogen and inlet that was heated to 50  $^\circ\text{C}$ . This purge vessel was further connected to an NO analyser (Sievers NOA 280i, Analytix, UK). A standard curve was produced by injecting 100  $\mu\text{L}$  of  $\text{NO}_2^-$  solutions (1000 nM, 500 nM, 250 nM, 125 nM, and 62.5 nM) and control sample containing deionised water. The area under the curve (AUC) for the latter was subtracted from the  $\text{NO}_2^-$  solutions to account for  $\text{NO}_2^-$  in the water used for dilutions. Following this, plasma samples were thawed in a water bath at 37  $^\circ\text{C}$  for 3 min and 100  $\mu\text{L}$  of the sample was injected into the purge vessel in duplicate. The concentration of NO cleaved during the reaction was then measured by the NO analyser. The AUC was calculated using Origin software (version 7) and divided by the gradient of the slope. The coefficient of variation for the measurement of  $[\text{NO}_2^-]$  in the current study was 3%.

For the measurement of plasma  $[\text{NO}_3^-]$ , vanadium reagent (32 mg of vanadium tri-chloride, 4 ml of 1M hydrochloric acid and 500  $\mu\text{L}$  of water) and 100  $\mu\text{L}$  of anti-foaming agent were placed into the glass purge vessel and heated to 95 °C. A standard curve was produced by injecting 25-50  $\mu\text{L}$  of  $\text{NO}_3^-$  solutions (100  $\mu\text{M}$ , 50  $\mu\text{M}$ , 25  $\mu\text{M}$ , 12.5  $\mu\text{M}$ , and 6.25  $\mu\text{M}$ ) and a control sample containing deionised water. Plasma samples were thawed and de-proteinised (200  $\mu\text{L}$  of sample, 400  $\mu\text{L}$  of zinc sulphate in deionised water at 10% weight/volume and 400  $\mu\text{L}$  of sodium hydroxide in deionised water at ratio of 1:1). Subsequently, 15-25  $\mu\text{L}$  of the sample was injected into the purge vessel in duplicate and plasma  $[\text{NO}_3^-]$  calculated as previously described for the  $\text{NO}_2^-$  assay. The coefficient of variation for the measurement of  $[\text{NO}_3^-]$  in the current study was 6%.

## 2.5. Determination of Plasma Volume Change

To determine the haematocrit, a small volume of venous blood was extracted into heparinised capillary tubes that were sealed at the distal end with a wax seal. The capillary tubes were then spun for 8 min at 15,000 revolutions/min in a micro-haematocrit centrifuge before the haematocrit was measured in triplicate using a Hawksey haematocrit reader. The coefficient of variation for the measurement of haematocrit in the current study was 0.4%. Haemoglobin concentration was determined using the Randox colorimetric method (RX Monza, Randox Laboratories, UK). Briefly, 20  $\mu\text{L}$  of whole blood was mixed in a cuvette with 2.5 ml of haemoglobin reagent before being incubated for 3 min at 25 °C. The haemoglobin concentration was determined by measuring absorbance when light at a wavelength of 546 nm was passed through the cuvette. The coefficient of variation for the measurement of haemoglobin in the current study was 2%. Total blood volume (TBV) and total PV (TPV) at baseline were estimated using the Nadler equations (Nadler et al. 1962):

$$\text{Males TBV} = (0.3669 \times \text{height in meters}^3) + (0.03219 \times \text{body mass in kilograms}) + 0.6041$$

$$\text{Females TBV} = (0.3561 \times \text{height in meters}^3) + (0.03308 \times \text{body mass in kilograms}) + 0.1833$$

$$\text{PV} = \text{TBV} * (1 - \text{Haematocrit})$$

The percentage change ( $\Delta$ ) in PV was estimated using the change in haematocrit and haemoglobin values using the method described by Dill and Costill (1974). The PV values for each time point are expressed as estimated TPV and the percentage  $\Delta$  from the baseline (0 min) sample (Fig. 4).

## 2.6. Statistical Analysis

All analyses were carried out using the Statistical Package for Social Sciences, Version 22 (SPSS Inc., Chicago, IL, USA). GraphPad Prism version 7 (GraphPad Software Inc., San Diego, USA) was used to create the figures. Data are expressed as the mean  $\pm$  standard deviation unless otherwise stated. The distribution of the data was tested using the Shapiro-Wilk test. A one-way repeated-measures ANOVA was used to examine the differences between measurement points  $[\text{NO}_3^-]$ ,  $[\text{NO}_2^-]$ , PV, HR and BP. *Post-hoc* analysis was used to determine the difference from the baseline and all other time points using a paired samples t-tests with Bonferroni correction for multiple pairwise comparisons. The association between absolute and  $\Delta$  plasma  $[\text{NO}_3^-]$ ,  $[\text{NO}_2^-]$ , and TPV values was determined using Pearson's

correlation coefficient. Statistical significance was declared when  $P < 0.05$ . Probability values are expressed with 95% confidence intervals (95% CI) where appropriate.

### 3. Results

#### 3.1. Nitrite

Baseline values of  $[\text{NO}_2^-]$  were significantly elevated in the BR trial compared to the CON trial ( $P < 0.01$ , 95% CI 154-320 nM, Fig. 2) and at all other time points. There was a significant main effect of measurement point on plasma  $[\text{NO}_2^-]$  in both trials ( $P < 0.001$ ). Plasma  $[\text{NO}_2^-]$  was not different to baseline after 10 min of lying supine in either condition (both  $P > 0.05$ ) but decreased significantly after 30 min (CON:  $P = 0.02$ , 95% CI 5-82 nM; BR:  $P < 0.01$ , 95% CI 39-213 nM). In the CON trial, plasma  $[\text{NO}_2^-]$  increased from the 30 min supine time point upon standing ( $P < 0.01$ , 95% CI 15-51 nM) and sitting ( $P < 0.01$ , 95% CI 32-76 nM) and remained higher following exercise ( $P < 0.05$ , 95% CI 7-89 nM). In the BR trial, plasma  $[\text{NO}_2^-]$  increased from 30 min supine upon standing ( $P < 0.01$ , 95% CI 35-98 nM) and sitting ( $P < 0.01$ , 95% CI 58-251 nM). Following exercise, plasma  $[\text{NO}_2^-]$  was significantly reduced compared to sitting ( $P = 0.02$ , 95% CI 13-180 nM) but was not different to the 30 min supine time point ( $P = 0.14$ ). Plasma  $[\text{NO}_2^-]$  was not correlated with TPV either for absolute (CON,  $R = 0.04$ ,  $P = 0.74$ ; BR,  $R = 0.03$ ,  $P = 0.78$ ) or  $\Delta$  values (CON,  $R = -0.12$ ,  $P = 0.31$ ; BR  $R = -0.11$ ,  $P = 0.39$ ).

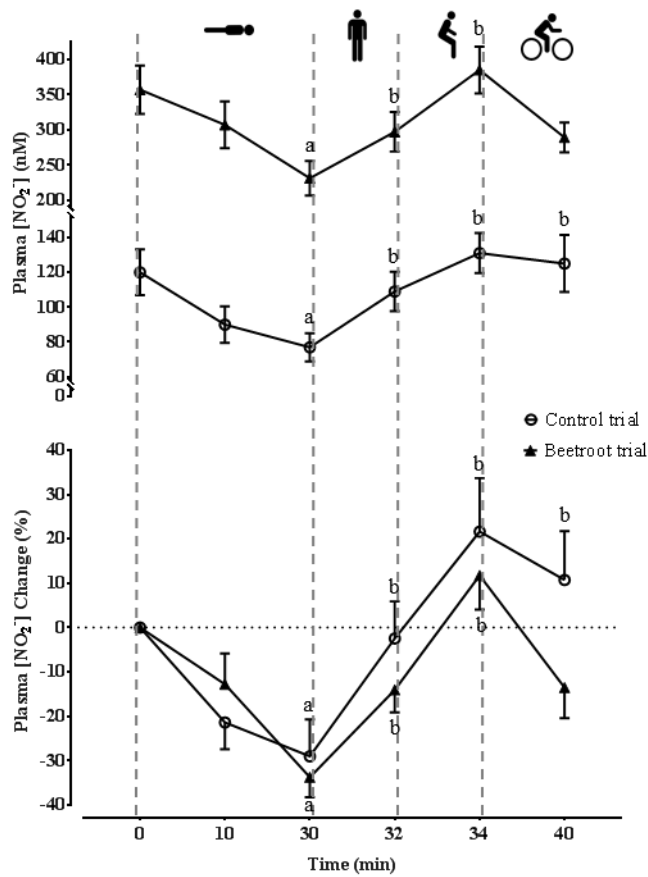


Fig. 2. Changes in mean  $\pm$  SEM plasma [NO<sub>2</sub><sup>-</sup>] expressed as absolute values (top) and percentage change from baseline (bottom). The graphical images at the top of the figure denote the supine, standing, seated, and exercise phases of the trial. a denotes a significant decrease compared to baseline ( $P < 0.05$ ). b denotes a significant increase compared to 30 min time point ( $P < 0.05$ ). All time points in the BR trial were significantly higher than the CON trial ( $P < 0.01$ ).

### 3.2. Nitrate

Plasma [NO<sub>3</sub><sup>-</sup>] at baseline was higher in the BR trial compared to CON ( $P < 0.01$ , 95% CI 395-561  $\mu$ M, Fig. 3) and at all other time points. There was a significant main effect of measurement point on plasma [NO<sub>3</sub><sup>-</sup>] in the CON trial ( $P < 0.01$ ) but not the BR trial ( $P = 0.20$ ). In the CON trial, plasma [NO<sub>3</sub><sup>-</sup>] was higher after exercise compared to 10 min of lying supine ( $P < 0.01$ ,

95% CI 4-24  $\mu\text{M}$ ) but was not different between any other measurement points. Plasma  $[\text{NO}_3^-]$  was not correlated with TPV either for absolute (CON,  $R=-0.10$ ,  $P=0.37$ ; BR,  $R=-0.18$ ,  $P=0.10$ ) or  $\Delta$  values (CON,  $R=-0.21$ ,  $P=0.08$ ; BR  $R=-0.02$ ,  $P=0.86$ ).

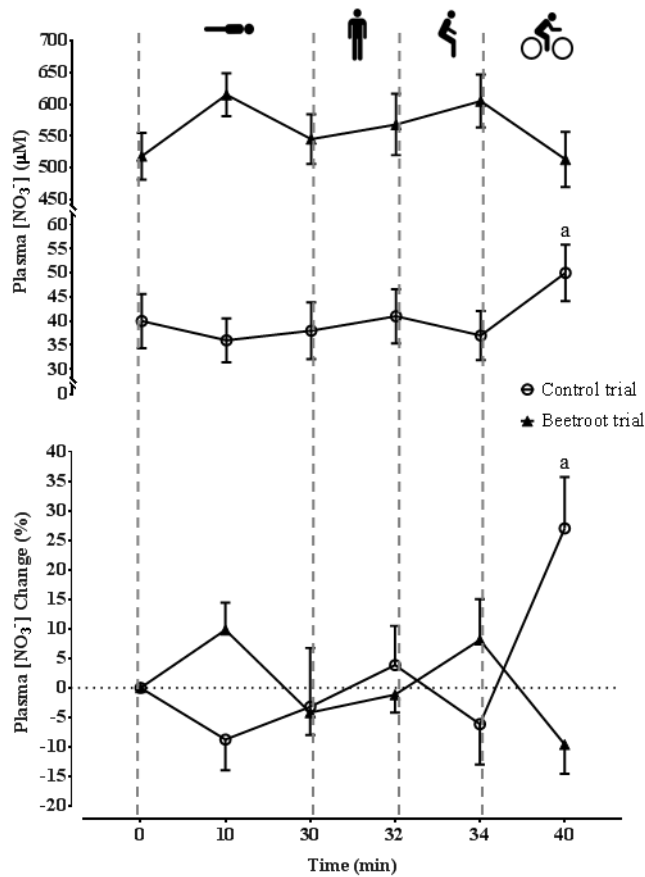


Fig. 3. Changes in mean  $\pm$  SEM plasma  $[\text{NO}_3^-]$  expressed as absolute values (top) and percentage change from baseline (bottom). The graphical images at the top of the figure denote the supine, standing, seated, and exercise phases of the trial. a denotes a significant increase compared to 10 min time point ( $P<0.01$ ). All time points in the BR trial were significantly higher than the CON trial ( $P<0.01$ ).

### 3.3. Plasma Volume

After 10 min of lying supine, PV increased from baseline in both conditions (all  $P<0.01$ , CON 95% CI 0.09-0.51 L, BR 95% CI 0.12-0.42 L, Fig. 4) and remained elevated at the 30 min measurement point (all  $P<0.05$ , CON 95% CI 0.04-0.63 L, BR 95% CI 0.15-0.53 L). PV then declined significantly from the 30 min supine measurement upon standing, sitting, and exercise in both conditions (all  $P<0.05$ ).

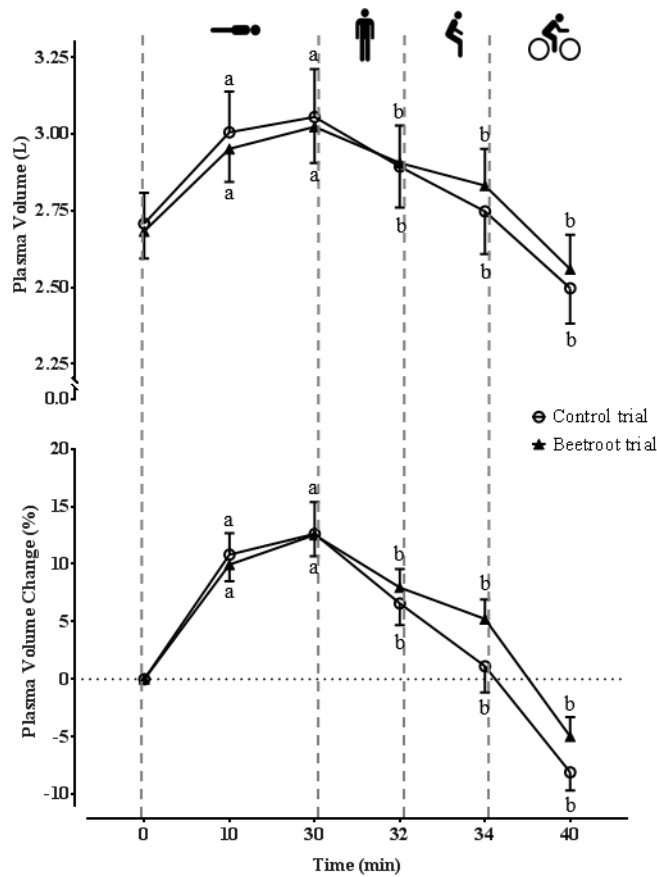


Fig. 4. Changes in mean  $\pm$  SEM plasma volume expressed as absolute values (top) and percentage change from baseline (bottom). The graphical images at the top of the figure denote the supine, standing, seated, and exercise phases of the trial. a denotes a significant increase compared to baseline ( $P<0.01$ ). b denotes a significant decrease compared to 30 min of laying supine ( $P<0.05$ ).

### 3.4. Blood Pressure

Baseline measurements of MAP were significantly lower in the BR trial compared to CON ( $P<0.01$ , 95% CI 1-5 mmHg, Fig. 5) but was not different at any other time point. There was a significant main effect of time on MAP in both conditions ( $P<0.01$ ). MAP was higher during standing compared to baseline (CON,  $P<0.01$ , 95% CI 4-13 mmHg; BR,  $P<0.01$ , 95% CI 4-16 mmHg) and following 25 min lying supine (CON,  $P<0.01$ , 95% CI 6-14 mmHg; BR,  $P<0.01$ , 95% CI 4-17 mmHg). In the CON trial, MAP was higher during sitting compared to lying supine for 25 min ( $P=0.04$ , 95% CI 0-10 mmHg). In the BR trial, MAP was higher during standing compared to sitting ( $P=0.03$ , 95% CI 0-7 mmHg).

### 3.5 Heart Rate

There was a significant main effect of time ( $P<0.01$ ) on HR in both the CON and BR trials. HR was typically higher during the standing and sitting phases compared to the supine time points (all  $P<0.01$ ). There was no difference in HR between sitting and standing measurements (both  $P>0.05$ ). In both conditions, post-exercise HR was higher than all other time points (all  $P<0.01$ ).



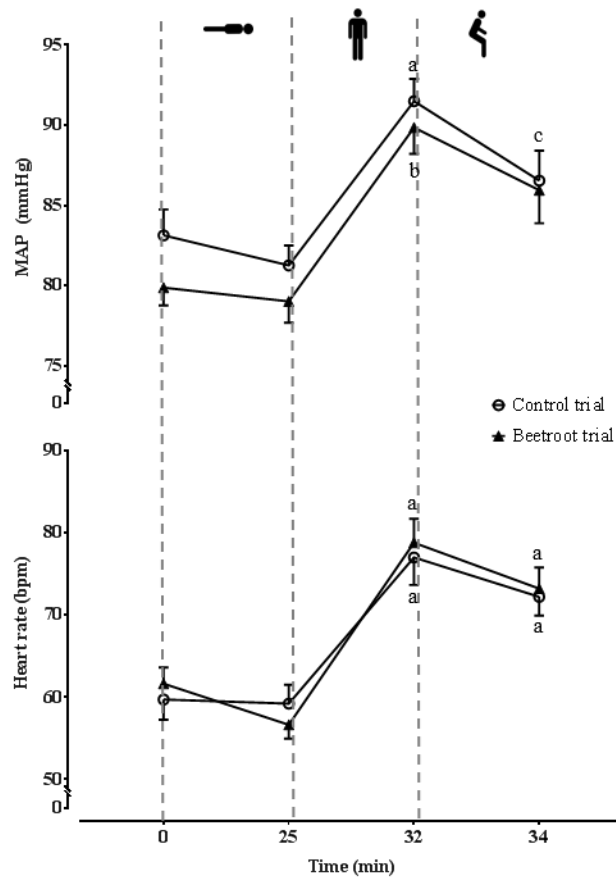


Fig. 5. Changes in mean  $\pm$  SEM mean arterial pressure (top) and heart rate (bottom) expressed as absolute values. The graphical images at the top of the figure denote the supine, standing, seated, and exercise phases of the trial. a denotes a significant increase compared to baseline and 25 min time point ( $P < 0.01$ ). b denotes a significant increase compared to all other time points ( $P < 0.05$ ). c denotes significant increase compared to 25 min time point ( $P < 0.05$ ). Baseline measurements of mean arterial pressure were lower in the BR trial compared to the CON trial ( $P < 0.01$ ).

#### 4. Discussion

To our knowledge, this study is the first to report that plasma  $[\text{NO}_2^-]$  is substantially altered by varying body posture while these changes have minimal impact on plasma  $[\text{NO}_3^-]$ . Here, we

report that plasma  $[\text{NO}_2^-]$  is increased during sitting and standing compared to lying supine which substantially extends our previous findings that plasma  $[\text{NO}_2^-]$  declines during a period of lying supine (Muggeridge et al. 2015).

As expected, moving between different body postures resulted in consistent, marked and rapid changes in PV. Following a period of standing upright, PV increased by ~10% after 10 min in the supine posture. There was a further increase in PV at the 30 min time point, although this was of a much smaller magnitude (~13% from baseline). Previous data suggests that PV stabilises approximately 20 min in the supine posture (Hagan et al. 1978) which is pertinent when measuring the concentration of blood metabolites in exercise studies. On the other hand, moving from supine to standing resulted in an almost immediate (~2 min) reduction in PV which reduced further as participants continued to the seated posture. Again, the magnitude of the response was profound, with PV dropping by 6-10% following brief periods of standing and sitting from the end of the supine phase. A short period of exercise caused a further large reduction in PV which corresponded to a decline of ~19% from the end of the supine phase. These data are broadly in line with values reported elsewhere in the literature (Hagan et al. 1978) although others (Hansen 1968; Lippi et al. 2015) have reported larger declines in PV during standing (~14%), likely due to longer period of time in this posture.

These postural-induced alterations in PV are readily explainable and the likely mechanisms have been known for some time (Thompson et al. 1928). Adopting a standing posture increases local hydrostatic pressure, particularly in the lower limbs, which forces fluid and some molecules from the intravascular to the interstitial space (Krogh et al. 1932). The augmented reduction in PV during exercise is most likely caused by an increased intra-capillary pressure

in the contracting muscles (Hansen 1968). These fluid shifts do eventually stabilise due to counter pressure exerted by the tissue and an increase in the intravascular oncotic pressure (Youmans et al. 1934). As evidenced in the present study, the reduction in circulating blood volume and venous return can lead to an increase in HR and BP. It is important to note, however, that standing may initially reduce BP and transient changes in this response may vary between individuals (Eşer et al. 2007).

For the first time, we demonstrate that plasma  $[\text{NO}_2^-]$  changes substantially between supine, standing and sitting phases. Where PV increases during the supine phase,  $[\text{NO}_2^-]$  decreases during lying supine and increases on standing and sitting. These data are perhaps not surprising given postural-induced PV shifts have previously been reported to alter the concentration of other constituents in the blood in a similar fashion (Thompson et al. 1928; Fawcett and Wynn 1960; Lippi et al. 2015). Consequently, it might be expected that a considerable proportion of the change in plasma  $[\text{NO}_2^-]$  can be accounted for by a “dilution effect” where, for example, an increased PV reduces the measured concentration of the number of  $\text{NO}_2^-$  particles. However, there was no correlation between either absolute or  $\Delta$  plasma  $[\text{NO}_2^-]$  and PV values suggesting plasma fluid shifts account for only a small proportion of the variance in  $[\text{NO}_2^-]$  during postural changes. Furthermore, plasma  $[\text{NO}_3^-]$  does not change uniformly as posture is altered and was not correlated with PV. Instead, it seems probable that postural-induced alterations in NO metabolism may account for these findings.

In line with previous research, both plasma  $[\text{NO}_2^-]$  and  $[\text{NO}_3^-]$  were considerably elevated by ingestion of  $\text{NO}_3^-$ -rich beetroot juice (198% and 1163%, respectively). However, given plasma  $[\text{NO}_2^-]$  is reported to peak ~2.5 h after acute ingestion of beetroot juice (Webb et al. 2008), it

is perhaps surprising that plasma  $[\text{NO}_2^-]$  in the present study declined during the supine phase of the BR trial to the same extent as CON (i.e. 2 – 2.5 h after ingestion). Nevertheless, we (McIlvenna et al. 2017) and others (James et al. 2015) have previously demonstrated that plasma  $\text{NO}_2^-$  pharmacokinetics following dietary  $\text{NO}_3^-$  ingestion appear to vary substantially between individuals. For example, Wylie and colleagues (2013a) reported that time taken for plasma  $[\text{NO}_2^-]$  to peak following administration of a similar dose of  $\text{NO}_3^-$ -rich beetroot juice ranged between 77 and 213 min. Therefore, it is plausible that while dietary-derived  $\text{NO}_2^-$  was still increasing in the plasma in some participants, it was declining in others.

Measurement of plasma  $[\text{NO}_2^-]$  and  $[\text{NO}_3^-]$  is further complicated by the fact that changes in posture also alter the rate of endogenous NO production. Shear stress is a frictional force exerted by blood moving across the endothelium and is reported to increase during standing compared to sitting (Morishima et al. 2017). Endothelial cells rapidly respond to shear stress with an acute increase in intracellular calcium that enhances the binding of calmodulin to eNOS and increases eNOS activity and NO production (Boo and Jo 2003; Rassaf et al. 2006). On the contrary, moving from a supine to a seated position has been demonstrated to reduce shear rate in young but not old participants (Trinity et al. 2015). Given that shear rate was not measured in the current study, we can only speculate as to how this may have impacted endogenous synthesis of NO and related metabolites. Conversion of NO to  $\text{NO}_3^-$  by heme proteins in the blood and tissues occurs fairly rapidly (Shiva et al. 2006), such that  $\text{NO}_3^-$  is considered to be the major breakdown product in the presence of sufficient amounts of  $\text{O}_2$  (Kelm 1999). Conversely, NO can be oxidised to  $\text{NO}_2^-$  via various oxidants in plasma and tissues (Shiva et al. 2006). It should also be noted that a considerable portion of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  are stored in tissues. In rodents, the liver, blood, and skeletal muscle contain equivalent amounts of  $\text{NO}_2^-$  (~0.5 – 0.7 nmol/g) whereas  $\text{NO}_3^-$  is considerably higher in muscle (~200 nmol/g) compared

to blood (~80 nmol/g) and the liver (~10 nmol/g) (Piknova et al. 2015). Furthermore, Nyakayiru et al. (2017) have recently shown that ingestion of sodium  $\text{NO}_3^-$  results in a substantial and sustained increase in muscle  $[\text{NO}_3^-]$ , with reported values exceeding those in plasma. However, muscle  $[\text{NO}_2^-]$  was below the detection limit both before and after  $\text{NO}_3^-$  supplementation. Therefore, when considering the impact of postural-induced fluid shifts in the context of measuring  $[\text{NO}_2^-]$  and  $[\text{NO}_3^-]$ , we must also factor in the change in endogenous NO production, the oxidation of NO to various metabolic endpoints, and the transfer of these metabolites to and from different tissues.

Data from this study also demonstrates that short-duration sub-maximal cycling exercise leads to a reduction in plasma  $[\text{NO}_2^-]$  in both conditions but a variable response in plasma  $[\text{NO}_3^-]$ . Although plasma  $[\text{NO}_3^-]$  did not differ statistically between time points overall, there was a substantial increase from pre- to post-exercise in the CON trial and a reduction in the BR trial. The reduction in plasma  $[\text{NO}_2^-]$  is consistent with some (Larsen et al. 2007; Kelly et al. 2014), but not all (Larsen et al. 2010) previous studies and potentially results from an increased conversion of  $\text{NO}_2^-$  to NO during exercise. Differences between study cohorts and the intensity and duration of the exercise protocols may explain the inconsistencies in these data. Cosby and colleagues (2003) suggest that  $\text{NO}_2^-$  is a major bioavailable pool of NO and present data demonstrating an increased reduction of  $\text{NO}_2^-$  to NO by deoxyhemoglobin during exercise. Furthermore, in animal studies, the initiation of exercise has been shown to increase the demand for NO and upregulate eNOS activity (Maiorana et al. 2003). Therefore, post-exercise changes in plasma  $[\text{NO}_2^-]$  and  $[\text{NO}_3^-]$  in dietary  $\text{NO}_3^-$  supplementation studies must be interpreted cautiously due to the aforementioned pharmacokinetics of these metabolites and the individual variability in the response (James et al. 2015; McIlvenna et al. 2017).

Although the precise mechanisms explaining the alterations in plasma  $[\text{NO}_2^-]$  remain unclear, the magnitude of the change in this outcome during the adoption of different postures highlights the importance of standardising posture in experimental trials where this outcome is important. Indeed, an inconsistent approach to the posture of participants during blood collection may at least partly explain why measurements of plasma  $[\text{NO}_3^-]$  are comparable between different studies in healthy participants while  $[\text{NO}_2^-]$  varies considerably. The present study is not without limitations as the phases of sitting, standing, and exercise were very brief and the fate of the ingested  $\text{NO}_3^-$  is impossible to determine without more advanced measurement methods. Furthermore, the order of the trials was not randomised and nor was there inclusion of a placebo condition that required the ingestion of a matched volume of  $\text{NO}_3^-$ -depleted beetroot juice. However, our data demonstrates the proportional change in PV,  $[\text{NO}_3^-]$  and  $[\text{NO}_2^-]$  were consistent between BR and CON conditions suggesting these experimental limitations do not diminish confidence in the findings. Notwithstanding, there are two primary recommendations that emanate from this work. Firstly, if differences in  $[\text{NO}_2^-]$  are to be compared either within or between participants in an experimental trial, participants should be lying supine for a standardised period of time before blood collection. For baseline measurements the supine period should be a minimum of 20 – 30 min. For post-exercise measurements the supine period should be brief but standardised. Secondly, the posture of participants during blood collection and the duration that this posture was maintained before blood collection should be clearly documented in research manuscripts to allow better comparison of data between studies.

## **5. Conclusion**

The principal finding from this study is that posture has a profound impact on the concentration of plasma  $\text{NO}_2^-$  regardless of whether  $[\text{NO}_2^-]$  was normal or elevated by dietary  $\text{NO}_3^-$  supplementation. The lack of correlation between PV and  $[\text{NO}_2^-]$  suggests that fluid shifts cannot solely account for this response. While postural alterations in shear stress and endogenous NO production may be contributing factors, we do not have experimental data to support this notion. Nevertheless, researchers should standardise the posture of participants at rest and post exercise when multiple blood samples are to be collected and fully document these procedures during dissemination of their data.

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## References

- Bailey SJ, Winyard P, Vanhatalo a., et al (2009) Dietary nitrate supplementation reduces the O<sub>2</sub> cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol* 107:1144–1155. doi: 10.1152/jappphysiol.00722.2009
- Boo YC, Jo H (2003) Flow-dependent regulation of endothelial nitric oxide synthase: role of protein kinases. *Am J Physiol Cell Physiol* 285:C499–C508. doi: 10.1152/ajpcell.00122.2003
- Castello PR, David PS, McClure T, et al (2006) Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: Implications for oxygen sensing and hypoxic signaling in eukaryotes. *Cell Metab* 3:277–287. doi: 10.1016/j.cmet.2006.02.011
- Cohn JN (1966) Relationship of plasma volume changes to resistance and capacitance vessel effects of sympathomimetic amines and angiotensin in men. *Clin Sci* 30:267–78.
- Cosby K, Partovi KS, Crawford JH, et al (2003) Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med* 9:1498–505. doi: 10.1038/nm954
- Dill DB, Costill DL (1974) Calculation of percentage changes in volumes of blood, plasma, and red cells in dehydration. *J Appl Physiol* 37:247–248. doi: ET0013
- Duncan C, Dougall H, Johnston P, et al (1995) Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat Med* 1:546–551. doi: 10.1038/nm0695-546
- Eşer İ, Khorshid L, Yapucu Güneş Ü, Demir Y (2007) The effect of different body positions on blood pressure. *J Clin Nurs* 16:137–140. doi: 10.1111/j.1365-2702.2005.01494.x
- Fawcett JK, Wynn V (1960) Effects of posture on plasma volume and some blood constituents. *J Clin Pathol* 13:304–310. doi: 10.1136/jcp.13.4.304
- Forstermann U, Sessa WC (2012) Nitric oxide synthases: Regulation and function. *Eur Heart J* 33:829–837. doi: 10.1093/eurheartj/ehr304
- Hagan RD, Diaz FJ, Horvath SM (1978) Plasma volume changes with movement to supine and standing positions. *J Appl Physiol* 45:414–417.
- Hagan RD, Diaz FJ, McMurray RG, Horvath SM (1980) Plasma volume changes related to posture and exercise. *Exp Biol Med* 165:155–160. doi: 10.3181/00379727-165-40952
- Hansen J (1968) The effect of short-term exercise on plasma volume and blood pressure in guanethidine-treated hypertensives. *Acta Med Scand* 183:553–558.
- Hsieh H-J, Liu C-A, Huang B, et al (2014) Shear-induced endothelial mechanotransduction: the interplay between reactive oxygen species (ROS) and nitric oxide (NO) and the pathophysiological implications. *J Biomed Sci* 21:3. doi: 10.1186/1423-0127-21-3
- Ignarro LJ, Buga GM, Wood KS, et al (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide (endothelium-dependent relaxation/vascular smooth muscle/cyclic GMP). *Med Sci* 84:9265–9269. doi: 10.1073/pnas.84.24.9265
- James PE, Willis GR, Allen JD, et al (2015) Nitrate pharmacokinetics: Taking note of the difference. *Nitric Oxide* 48:44–50. doi: 10.1016/j.niox.2015.04.006



- Kargotich S, Goodman C, Keast D, Morton AR (1998) The influence of exercise-induced plasma volume changes on the interpretation of biochemical parameters used for monitoring exercise, training and sport. *Sport Med* 26:101–117. doi: 10.2165/00007256-199826020-00004
- Kelly J, Vanhatalo A, Bailey SJ, et al (2014) Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O<sub>2</sub> uptake dynamics during exercise in hypoxia and normoxia. *Am J Physiol Regul Integr Comp Physiol* 920–930. doi: 10.1152/ajpregu.00068.2014
- Kelm M (1999) Nitric oxide metabolism and breakdown. *Biochim Biophys Acta - Bioenerg* 1411:273–289. doi: 10.1016/S0005-2728(99)00020-1
- Krogh A, Landis EM, Turner AH (1932) The movement of fluid through the human capillary wall in relation to venous pressure and to the colloid osmotic pressure of the blood. *J Clin Invest* 11:63–95. doi: 10.1172/JCI100408
- Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B (2007) Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol* 191:59–66. doi: 10.1111/j.1748-1716.2007.01713.x
- Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B (2010) Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free Radic Biol Med* 48:342–347. doi: 10.1016/j.freeradbiomed.2009.11.006
- Lauer T, Preik M, Rassaf T, et al (2001) Plasma nitrite rather than nitrate reflects regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. *Proc Natl Acad Sci U S A* 98:12814–12819. doi: 10.1073/pnas.221381098
- Lippi G, Salvagno GL, Lima-Oliveira G, et al (2015) Postural change during venous blood collection is a major source of bias in clinical chemistry testing. *Clin Chim Acta* 440:164–168. doi: 10.1016/j.cca.2014.11.024
- Lundberg JO, Govoni M (2004) Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med* 37:395–400. doi: 10.1016/j.freeradbiomed.2004.04.027
- Lundberg JO, Weitzberg E (2009) NO generation from inorganic nitrate and nitrite: Role in physiology, nutrition and therapeutics. *Arch Pharm Res* 32:1119–1126. doi: 10.1007/s12272-009-1803-z
- Lundberg JO, Weitzberg E, Gladwin MT (2008) The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov* 7:156–167. doi: 10.1038/nrd2466
- Lundberg JO, Weitzberg E, Lundberg JM, Alving K (1994) Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* 35:1543–1546. doi: 10.1136/gut.35.11.1543
- Maiorana A, O’Driscoll G, Taylor R, Green D (2003) Exercise and the nitric oxide vasodilator system. *Sport Med* 33:1013–1035. doi: 10.2165/00007256-200333140-00001
- McIlvenna LC, Monaghan C, Liddle L, et al (2017) Beetroot juice versus chard gel: A pharmacokinetic and pharmacodynamic comparison of nitrate bioavailability. *Nitric Oxide - Biol Chem* 64:61–67. doi: 10.1016/j.niox.2016.12.006
- McMahon NF, Leveritt MD, Pavey TG (2017) The effect of dietary nitrate supplementation on endurance exercise performance in healthy adults: A systematic review and meta-

- analysis. *Sport. Med.* 47:735–756.
- Millar TM, Stevens CR, Benjamin N, et al (1998) Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. *FEBS Lett* 427:225–228. doi: 10.1016/S0014-5793(98)00430-X
- Modin A, Björne H, Herulf M, et al (2001) Nitrite-derived nitric oxide: A possible mediator of “acidic-metabolic” vasodilation. *Acta Physiol Scand* 171:9–16. doi: 10.1046/j.1365-201X.2001.171001009.x
- Morishima T, Restaino RM, Walsh LK, et al (2017) Prior exercise and standing as strategies to circumvent sitting-induced leg endothelial dysfunction. *Clin Sci* 131:1045–1053. doi: 10.1042/CS20170031
- Muggeridge DJ, Sculthorpe N, Grace FM, et al (2015) Acute whole body UVA irradiation combined with nitrate ingestion enhances time trial performance in trained cyclists. *Nitric Oxide* 48:3–9. doi: 10.1016/j.niox.2014.09.158
- Nadler SB, Hidalgo JU, Bloch T, Nadler S.B., Hidalgo J.U. BT (1962) Prediction of blood volume in normal human adults. *Surgery* 51:224–32.
- Nyakayiru J, Kouw IWK, Cermak NM, et al (2017) Sodium nitrate ingestion increases skeletal muscle nitrate content in humans.
- Pawlak-Chaouch M, Boissière J, Gamelin FX, et al (2016) Effect of dietary nitrate supplementation on metabolic rate during rest and exercise in human: A systematic review and a meta-analysis. *Nitric Oxide* 53:65–76. doi: 10.1016/j.niox.2016.01.001
- Pelletier MM, Kleinbongard P, Ringwood L, et al (2006) The measurement of blood and plasma nitrite by chemiluminescence: Pitfalls and solutions. *Free Radic Biol Med* 41:541–548. doi: 10.1016/j.freeradbiomed.2006.05.001
- Piknova B, Park JW, Swanson KM, et al (2015) Skeletal muscle as an endogenous nitrate reservoir. *Nitric Oxide* 15:10–16. doi: 10.1016/j.bbamem.2015.02.010.Cationic
- Rassaf T, Heiss C, Hendgen-Cotta U, et al (2006) Plasma nitrite reserve and endothelial function in the human forearm circulation. *Free Radic Biol Med* 41:295–301. doi: 10.1016/j.freeradbiomed.2006.04.006
- Restaino RM, Walsh LK, Morishima T, et al (2016) Endothelial dysfunction following prolonged sitting is mediated by a reduction in shear stress. *Am J Physiol Heart Circ Physiol* ajpheart.00943.2015. doi: 10.1152/ajpheart.00943.2015
- Rogers SC, Khalatbari A, Gapper PW, et al (2005) Detection of human red blood cell-bound nitric oxide. *J Biol Chem* 280:26720–26728. doi: 10.1074/jbc.M501179200
- Sandbakk SB, Sandbakk O, Peacock O, et al (2015) Effects of acute supplementation of L-arginine and nitrate on endurance and sprint performance in elite athletes. *Nitric Oxide* 48:10–15. doi: 10.1016/j.niox.2014.10.006
- Sessa WC (2004) eNOS at a glance. *J Cell Sci* 117:2427–2429. doi: 10.1242/jcs.01165
- Shiva S, Wang X, Ringwood L a, et al (2006) Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. *Nat Chem Biol* 2:486–493. doi: 10.1038/nchembio813
- Siervo M, Lara J (2013) Inorganic nitrate and beetroot juice supplementation reduces blood pressure in adults: a systematic review and meta-analysis. *J Nutr* 143:818–826. doi:

10.3945/jn.112.170233.tonically

- Stokke KT, Rootwelt K, Wergeland R, Vale JR (1986) Changes in plasma and red cell volumes during exposure to high altitude. *Scand J Clin Lab Invest Suppl* 184:113–7.
- Thompson WO, Thompson PK, Dailey ME (1928) The effect of posture upon the composition and volume of the blood in man. *J Clin Invest* 5:573–604. doi: 10.1172/JCI100179
- Totzeck M, Hendgen-Cotta UB, Rammos C, et al (2012) Higher endogenous nitrite levels are associated with superior exercise capacity in highly trained athletes. *Nitric Oxide - Biol Chem* 27:75–81. doi: 10.1016/j.niox.2012.05.003
- Trinity JD, Groot HJ, Layec G, et al (2015) Impact of age and body position on the contribution of nitric oxide to femoral artery shear rate: Implications for atherosclerosis. *Hypertension* 63:1019–1025. doi: 10.1161/HYPERTENSIONAHA.113.02854.Impact
- Uematsu M, Ohara Y, Navas JP, et al (1995) Regulation of endothelial cell nitric oxide synthase mRNA expression by shear stress. *Am J Physiol Cell Physiol* 269:C1371–1378.
- Webb AJ, Patel N, Loukogeorgakis S, et al (2008) Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension* 51:784–790. doi: 10.1161/HYPERTENSIONAHA.107.103523
- Wylie LJ, Kelly J, Bailey SJ, et al (2013a) Beetroot juice and exercise: pharmacodynamic and dose-response relationships. *J Appl Physiol* 115:325–336. doi: 10.1152/jappphysiol.00372.2013
- Wylie LJ, Mohr M, Krstrup P, et al (2013b) Dietary nitrate supplementation improves team sport-specific intense intermittent exercise performance. *Eur J Appl Physiol* 113:1673–1684. doi: 10.1007/s00421-013-2589-8
- Youmans JB, Wells HS, Donley D, et al (1934) The Effects of Posture (Standing) on the Serum Protein Concentration and Colloid Osmotic Pressure of Blood from the Foot in Relation to the Formation of Edema. *J Clin Invest* 13:447–459.